Molecular approaches to diagnostics for plant parasitic nematodes of biosecurity concern

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By

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Declaration

I declare that this is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institute.

Matthew Ngee Hock Tan

"Scientific achievement doesn't rest. It's a lifetime endeavour. And if you want to know the future, create it."

Anonymous, 2010

Abstract

Nematode identification by classical methods is a highly skilled undertaking, in which trained taxonomists examine samples microscopically and identify nematodes using keys based on morphological details. The accuracy of diagnosis depends considerably on the skill of the taxonomist. More recently, molecular diagnostic techniques have been developed to identify such nematodes, and the focus of this thesis is the development and application of new approaches to nematode diagnostics. The species studied included root lesion nematodes (RLNs, *Pratylenchus* spp.), cyst nematodes (CNs *Heterodera* and *Globodera* spp.), and the pine wood nematode (PWN, *Bursaphelenchus xylophilus*). Six populations of four species of RLNs isolated from wheat and sorghum plants were maintained on carrot pieces *in vitro* for the work. Similarly, seventeen populations of five species of CNs were also studied: in some cases for biosecurity species (eg for soybean and potato cyst nematodes, and PWN) the materials had either to be studied overseas or extracts obtained from overseas for analysis.

For nucleic acid based diagnostics, an ITS-based PCR approach was used to identify different species of RLNs and study sequence differences within and between different species and populations. Phylogenetic trees were constructed to compare the data generated in this thesis with those of published sequences for the nematodes studied. The results obtained showed that there were relatively small differences in sequence between different populations of a individual species, but significantly greater differences between species. Comparing ITS sequences of different RLN species, that of *P. neglectus* was 58% similar to that of *P. thornei,* and compared to that of *P. penetrans* and *P. zeae*, the similarity was 60% and 53% respectively. Similarly, comparing ITS sequences, *P. penetrans* was more closely related to *P. neglectus* (60%) than to *P. thornei* (59%) and *P. zeae* (58-59%).

For CNs, for the genus *Heterodera*, the similarity in ITS sequence of *H. schachtii* and *H. glycines* was 97-98%. When comparing *H. schachtii* and cereal cyst nematodes (CCNs), the similarity in ITS sequences was less, at 77-78% for *H. avenae*. *H. glycines*, a biosecurity listed pathogen, is not present in Australia and DNA from a Japanese population was obtained and sequenced. Based on the close sequence similarity of ITS regions (97-98%) between *H. schachtii* and *H. glycines*, it is suggested that *H. schachtii* can be use as a model for detecting future incursions of *H. glycines*. Of the three species of CCNs studied (*H. avenae*, *H. latipons* and *H. filipjevi)*, the two species, *H. latipons* and *H. filipjevi,* are also not present in Australia, and so published sequence data for these species was used for comparisons. ITS sequences of Australian *H. avenae* populations were compared to those of *H. latipons* and *H. filipjevi*: which were 86-88% and 94-96% similar to those of *H. avenae*. In the absence of the two biosecurity pathogens, *H. avenae* can be used as a suitable model to develop methods to detect the other

two CCN species. For the genus *Globodera*, a comparison of ITS sequences of *G. rostochiensis* (potato cyst nematode) populations from New Zealand and Japan differed by 2% and 3% from the consensus world collection of *G. rostochiensis* ITS sequences. These differences might reflect different routes of introduction of potato into Japan and New Zealand.

It was also noted that, in some cases, PCR analysis can lead to mis-identification. For example, in this study, a RLN from Queensland was identified by classical taxonomy as *P. zeae*, but when DNA sequencing was undertaken for this population, the resulting ITS sequence obtained was more similar to database sequences identified as *P. bolivianus* than to *P. zeae*. Such differences may result from initial mis-identification of the original sample, or from sequencing error.

Protein profiling was used as an alternative approach to ITS-based PCR identification of plant nematodes. This involves separating nematode proteins using Matrix Assisted Laser Desorption Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS) to generate diagnostic protein profiles. Protein profiling by MALDI-TOF MS was developed as a novel, rapid (<2hr) approach to identify plant-parasitic nematodes. Methods were developed to extract and analyse protein spectra by MALDI-TOF MS to identify nematode species of local and biosecurity concern. Protein profiles were generated for *H. glycines*, *G. rostochiensis, H. schachtii* and *H. avenae,* and for the RLNs, *P. neglectus, P. zeae, P. thornei* and *P. penetrans*. Diagnostic species-specific protein peaks were identified in the profiles for each species. The results obtained show that protein profiling using MALDI-TOF MS is a valid and rapid method for identifying plant nematodes, and the database provided here represents the most comprehensive resource for protein-based diagnostics of plant nematodes.

Two dimensional protein gel electrophoresis (2DE) was also assessed as a tool to analyse RLN and CN proteins in more detail. Nematode proteins were extracted and separated in a protein extraction buffer first by isoelectric point (pH range 3-10, 16.5% polyacrylamide gel) , followed by size separation using SDS gel electrophoresis, and gels stained with silver nitrate. The protein spots in the second dimension were recorded using a high resolution gel scanner. Ninety three distinct protein spots were found for *Pratylenchus* spp. and 89 distinct spots for *Heterodera* spp. Differences in the protein profiles between populations of one species, and between different species, were identified readily using 'Progenesis SameSpots' software. Thirteen protein spots for RLNs and nine spots for CNs were further analysed and characterised. From these, two significant proteins were identified as useful biomarkers that were present in two different populations of *P. neglectus* and one significant biomarker was identified present in *P. penetrans*. Similarly, specific differences in protein profiles were found within populations of

H. schachtii species which provided biomarkers that identified the different populations. For *H. schachtii* and *H. avenae*, two distinct protein spots were chosen as potential species specific biomarkers since they were present in three *H. schachtii* populations, and three species-specific biomarkers were chosen as specific to *H. avenae*.

Individual protein biomarkers were excised and sequenced after trypsin digestion to release peptides from the gel. The *m/z* ratio of the peptides fragments were then analyses by MALDI-TOF MS, and the pattern of fragmented peptides was then compared using blastP with those recorded on Mascot and NCBI databases to identify proteins from which the peptides were derived. Identified proteins included RutC family protein C23G10.2, major sperm protein, probable arginine kinase, ATP synthase subunit alpha-mitochondrial, glyceraldehyde-3-phosphate dehydrogenase 2 and vacuolar H atpase protein 8, protein C14C10.2b, Y20F4.3 transcript:Y20F4.3, protein 19C07, arabinogalactan endo-1,4- β -galactosidase 2, heat shock 70 kDa protein C, annexin 4F01, β-1, 4-endoglucanase 1 precursor, aldolase, cathepsin L and pectate lyase 1. Although it is not necessary to identify the function of diagnostic proteins, such additional information is useful. The value of identified species-specific proteins is their potential be use to develop antibody-based diagnostic tests, such as Lateral Flow Devices (LFDs), in which antibodies raised against specific biomarker proteins can be developed to provide a rapid field-based method for nematode identification.

Another nucleic acid based approach was also investigated, which also made use of ITS sequence data – termed anti-primer quantitative PCR (aQPCR) technology, using a qPCR equipment platform. In this approach an additional 'anti-primer' was added to fluorescent QPCR reactions (specific primers labeled with FAM, Cy5 or TET), which binds to and quenches unbound fluorescent label. This approach decreases background fluorescence and so increases accuracy of the diagnostic test, and was developed to provide a multiplex high-throughput assay for nematode diagnostics. With different fluorescence labels to tag different primers specific to different RLN species (*P. neglectus*, *P. penetrans* and *P. thornei*), the results obtained successfully differentiated these three species in a multiplex system, in a total reaction time of 2.5 hours. For this approach, the number of detectable species depends on the number of different fluorescent channels the qPCR machine can detect.

A further diagnostic procedure was also developed to try to increase the number of samples that could be analysed at one time. This procedure was termed 'Multiplex anti-primer denaturation PCR' (MAD PCR). It was derived by combining aQPCR technology and 'auto-sticky' PCR. This approach makes use of 'C^3 linkers' (or 'blocks') in primers, which prevent further PCR copying of the strand in which they occur, resulting in an overhang. With C^3 linkers inserted at

different positions in different primers, the qPCR was developed into a multiplex high-throughput assay. Different C^3 positioning on the primer results in different melt temperatures in a melt analysis after PCR. The difference in melt temperatures enabled differentiation between different C^3 inserted primers. To extend this technology, different fluorescent labels (such as FAM) were combined with different C^3 positions for primers specific to different species. After PCR, melt analysis was done to differentiate the species by the different temperatures at which melting of dsDNA occurred, as followed by changes in fluorescence with changing temperature. In the work undertaken C^3 blocks were incorporated in different primers to detect three different RLNs, ie a triplex system. The analysis of up to 12 species per qPCR could be done with the system as developed here, but with further refinement, and incorporating additional fluorescence labels with the 'triplex' system, 18 species could be detected using 6 channels.

As for other soil-borne disease agents, nematodes are usually extracted from plant tissue or soil samples for detection. The time taken to extract them is usually several days. In the final Results chapter extraction of DNA from nematode infected soil samples was combined with molecular identification, and developed as a potential package for a rapid nematode diagnostics in a field situation. To do this, a rapid isolation method was developed and termed 'DNA isolation rapid technique from soil'- 'DIRT(s)'. This involved extraction of DNA from nematode-infected soil samples using a customised blender (time taken about 2 min) and DNA capture column, followed by elution from the column and aQPCR analysis: the whole procedure took only 4 hours. Using the DIRT(s) technique and aQPCR technology, three different RLN species (*P. neglectus*, *P. thornei* and *P. penetrans*) infecting wheat plants were successfully identified after DIRT(s) extraction from soil using aQPCR in a multiplex assay.

The results are discussed in relation to current techniques used for nematode diagnostics. It is suggested that both protein-based and the novel PCR-based technologies (aQPCR, MAD PCR), and the new soil extraction method (DIRT(s)), can be developed to provide useful new approaches to detect and diagnose plant nematodes in biosecurity applications.

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Presentations

Some of the results provided in this thesis have been presented at the following scientific meetings.

Oral Presentations

Chapters 3 and 4:
CRC for National Plant Biosecurity Science Exchange 2011, Adelaide, Australia, 9 Feb 2011-11 Feb 2011. *Novel diagnostic identification of* plant-parasitic *nematodes - are they better and faster?*

Chapters 3 and 4:
Australasian Plant Pathology Society Symposium 2010, Perth, Australia, Oct 2010. *Novel molecular diagnostic framework for* plant-parasitic *nematode pests of biosecurity concern - better and faster than classical taxonomy?*

Chapter 6:

CRC for National Plant Biosecurity Science Exchange 2012, Perth, Australia, May 2012. *Using 'anti-primer' technology for nematode diagnostics.*

Australasian Plant Pathology Society Symposium 2011, Perth, Australia, Oct 2011. *Using 'anti-primer' technology for nematode diagnostics.* Awarded Best Student presentation.

International presentation

Tan, Matthew, Berryman, David, and Jones, Michael G.K. (2012). *Molecular approaches to diagnostics for plant parasitic nematodes of biosecurity concern*. Proc. 31st Int. Symp. of the European Society of Nematologists, Adana, Turkey, Sept 2012, p 62.

Poster Presentations

Chapters 3 and 4:
Tan M. N. H., Vanstone V. A., Goto D. and Jones M. G. K. (2010). "Molecular diagnostics of plant-parasitic nematodes identified as biosecurity risks," 20th Annual Combined Biological Sciences Meeting (University of Western Australia, Australia) Page 77. Awarded Combined Biological Sciences Meeting (CBSM) Plant Sciences Award.

List of abbreviations

BCN	beet cyst nematode
CCN	cereal cyst nematode
CNs	cyst nematodes
PWN	pine wood nematode
PCN	potato cyst nematode
RKN	root-knot nematodes
RLN	root lesion nematode
SCN	soybean cyst nematode
CHAPS	3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate
TET	5-Tetrachlorofluorescein
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside
FAM	6-Carboxyfluorescein
AFLP	amplified fragment length polymorphism
AP	'Anti-Primer'
aQPCR	anti-prime quantitative PCR
bp	basepair
BHQ-1	Black Hole Quencher-1
BSA	bovine serum albumin
ca	close approximate
Cy5	cyanine 5
C_T value	cycle threshold value
cv	cultivar
Da	Dalton
°C	degree Celsius
DNA	deoxyribose nucleic acid
dNTP	deoxyribo nucleotide tri-phosphate (mix of A, C, G + T)
DAFWA	Department of Agriculture and Food, Western Australia
DEEDI (QLD)	Department of Employment, Economic Development and Innovation, Queensland
dF/dT	derivative of the fluorescence as a function of temperature
DTT	dithiothreitol
Na_2EDTA	disodium ethylenediamine tetraacetate
dsDNA	double stranded deoxyribose nucleic acid
EM	electron microscopy
ELISA	Enzyme-linked immunosorbent assay
EDTA	ethylene diamine tetra-acetic acid
EU	European Union
fmole	femtomole
J1	first stage juvenile
J4	fourth stage juvenile
g	gram
hr (s)	hour (s)
HCl	hydrochloric acid
3'	hydroxyl-terminus of DNA molecule
IPG strip	immobilized ph gradient strip
IDT	Integrated DNA Technologies
ITS	internal transcribed spacers
IEF	isoelectric focusing
IPTG	Isopropyl-β-D-thio-galactoside
Jap	Japan

kV	kilovolt
kVh	kilovolt-hour
LSU	large subunit gene
LFD	lateral flow device
LAMP	loop-mediated isothermal amplification
LB medium	Luria Bertani medium
MAP	Multiplex 'Anti-Primer'
MAD PCR	Multiplex 'Anti-primer' Denaturation Polymerase Chain Reaction
$MgCl_2$	magnesium chloride
MS	mass spectrometry
MALDI-TOF	matrix-assisted laser desorption/ionisation-time of flight
µ	micro
µg	microgram
µL	microlitre
µm	micrometre
mg	milligram
mL	millilitre
mM	millimolar
min	minute
COI	mitochondrial cytochrome oxidase subunit I
mtDNA	mitochondrial DNA
M	molar
MEGA	molecular evolutionary genetic analysis
MAb	monoclonal
Ns	nanosecond
NJ	Neighbour Joining
NZ	New Zealand
1DE	One-dimensional electrophoresis
1-D gel	One dimensional gel
per. comm.	personal communication
PMSF	phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride
5'	phosphate- terminus of DNA molecule
PCR	polymerase chain reaction
KCl	potassium chloride
qPCR	quantitative polymerase chain reaction
QLD	Queensland
QDPI & F	Queensland Department of Primary Industries and Fisheries
RFLP	restriction fragment length polymorphism
rpm	revolutions per minute
RNA	ribonucleic acid
rDNA	ribosomal DNA
rRNA	ribosomal ribonucleic acid
SEM	scanning electron microscopy
sec	second
J2	second stage juvenile
SA	sinapinic acid
SSU	small subunit gene
NaOAc	sodium acetate
NaCl	Sodium Chloride
SDS	sodium dodecyl sulfate
SA	South Australia
SABC	State Agricultural Biotechnology Centre
SARDI	South Australian Research and Development Institute
SEB	Soil Extraction Buffer
J3	third stage juvenile

C^3	three carbon spacer
3-D	three dimensional
Taq	*Thermus aquaticus* DNA polymerase
TEM	Transmission electron microscopy
TFA	trifluoroacetic acid
TAE buffer	tris- acetic acid- EDTA electrophoresis buffer
Tris-HCL	tris- hydrochloric acid
2DE	Two-dimensional electrophoresis
2-D gel	Two dimensional gel
2-D PAGE	Two-Dimension Polyacrylamide Gel Electrophoresis
UV	ultraviolet
x g	unit for relative centrifugal force
V	volt
WA	Western Australia
WLB	worm lysis buffer

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